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Discovery of an artificial peptide agonist to the fibroblast growth factor receptor 1c/βKlotho complex from random peptide T7 phage display

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ABSTRACT

Fibroblast growth factor receptor-1c (FGFR1c)/βKlotho (KLB) complex is a receptor of fibroblast growth factor 21 (FGF21). Pharmacologically, FGF21 shows anti-obesity and anti-diabetic effects upon peripheral administration. Here, we report the development of an artificial peptide agonist to the FGFR1c/KLB heterodimer complex. The peptide, F91-8A07 (LPGRTCREYPDLWWVRCY), was discovered from random peptide T7 phage display and selectively bound to the FGFR1c/KLB complex, but not to FGFR1c and KLB individually. After subsequent peptide dimerization using a short polyethyleneglycol (PEG) linker, the dimeric F91-8A07 peptide showed higher potent agonist activity than that of FGF21 in cultured primary human adipocytes. Moreover, the dimeric peptide led to an expression of the early growth response protein-1 (Egr-1) mRNA in vivo, which is a target gene of FGFR1c. To the best of our knowledge, this is the first report of a FGFR1c/KLB complex-selective artificial peptide agonist.

1. Introduction

Receptor tyrosine kinase (RTK) is one of the common drug targets. In the past decade, peptides mimicking the function of a natural RTK ligand have been studied. The most successful example is erythropoietin (EPO) mimicking peptide: hematide [1–4]. It was discovered from random peptide phage display and has no sequence similarity with EPO. Peptide display technologies, such as phage display, ribosome display, and mRNA display, have been powerful tools in identifying novel target-binding peptides [5–7]. In recent years, Suga lab of Tokyo University reported an artificial Met agonist that was discovered from mRNA display [8]. On the other hand, there are few reported artificial agonists to RTKs, indicating the difficulty in generation of such peptides.

FGFR1c is one of RTKs and a receptor of FGF21. The pharmacological actions of FGF21 are as follows: anti-obesity effects, achieved by increasing energy expenditure to reduce body weight; anti-diabetic effects, achieved by decreasing blood glucose, insulin, triglycerides, LDL, and cholesterol levels; and improving insulin sensitivity and hepatic steatosis [9–11]. FGFR1c is activated by canonical FGFs through receptor-homodimerization. However, unlike canonical FGFs, FGF21 is not able to interact directly with FGFR1c. It depends on KLB as a co-receptor for interaction with FGFRs for their activation [12–14]. Recent studies suggested that the C-terminus of FGF21 binds directly to KLB, and the N-terminus is important to activate FGFR1c, but the detailed mechanism of receptor activation by FGF21 is not fully understood [15–17].

In this study, we hypothesized that acceleration/stabilization ability of FGFR1c/KLB complex formation and dimerization ability of FGFR1c would be required for molecules that have FGF21-like agonist activity. To test these two features, we searched for FGFR1c/KLB complex-selective peptide from random peptide T7 phage display. We isolated one phage clone that selectively binds to FGFR1c/KLB complex, but not to FGFR1c and KLB individually. After subsequent peptide dimerization by a chemical linker, we successfully generated an artificial peptide agonist to FGFR1c/KLB.

Abbreviations: FGFR, fibroblast growth factor receptor; KLB, βKlotho; FGF21, fibroblast growth factor 21; PEG, polyethyleneglycol; Egr-1, early growth response protein-1; RTK, receptor tyrosine kinase.

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2. Material and methods

2.1. Preparation of FGFR1c/KLB-Fc heterodimer

The plasmids for protein preparation were kindly gifted from National Institute of Advanced Industrial Science and Technology (AIST, Tsukuba, Japan). A stable mammalian cell line expressing human KLB(1-994)-V5-His-Fc was established from FreeStyle293 (Thermo Fisher Scientific, MA, USA) cells transfected with pcDNA3.1/human KLB(1-994)-V5-His-Fc, using G418 as a selection marker. To express human KLB(1-994)-V5-His-Fc and human FGFR1Cv2(1-376)-Fc heterodimer, pcDNA3.1/human FGFR1Cv2(1-376)-Fc were transfected into the cells expressing human KLB(1-994)-V5-His-Fc. Human KLB(1-994)-V5-His-Fc/human FGFR1Cv2(1-376)-Fc heterodimer was purified from the culture supernatant by NiNTA Superflow cartridge (QIAGEN, Hilden, Germany) affinity chromatography and HiLoad 26/60 Superdex 200 pg (GE Healthcare, Chalfont St. Giles, UK) gel filtration chromatography.

2.2. Phage library construction and panning

The T7 phage libraries displaying random peptides were constructed using mixed-oligonucleotides as template DNA and T7 Select 10-3 vector from Merck Millipore (Darmstadt, Germany), according to the methods described previously [5–7]. Recombinant Fc-fused protein was immobilized to Dynabeads Protein A/G (Invitrogen, CA, USA) with 0.5% BSA in PBS. After washing the beads by PBS containing 0.1% Tween20 (PBST), the beads were incubated with phage library for 1 h and were washed with PBST. The bound phages were eluted by 1% SDS and were introduced in *Escherichia coli* BLT5615 cells (Merck Millipore, Darmstadt, Germany) in log-phase growth for phage amplification. After bacteriolysis, the phages were recovered from the culture supernatant by centrifugation and PEG-precipitation. The recovered phages were suspended in PBS and used for the next round of panning.

2.3. Dimerization of F91-8A07 peptide with a chemical linker

Peptide and triethylamine (12 equivalents) was dissolved in DMF. To the solution of peptide 0.1 M 4-dimethylaminopyridine in DMF (0.1 equivalents) and a chemical linker, Fmoc-N-(PEG3-OSu)$_2$ (bis(2,5-dioxopyrrolidin-1-yl) 10-(((9H-fluoren-9-yl)methoxy) carbonyl)-4,7,13,16-tetraoxa-10-azanonadecane-1,19-dioate) (M03436, Watanabe Chemical Instituted, Hiroshima, Japan) or Fmoc-N-(PEG6-OSu)$_2$ (bis(2,5-dioxopyrrolidin-1-yl) 19-(((9H-fluoren-9-yl)methoxy)carbonyl)-4,7,10,13,16,22,25,28,31,34-decaoxa-19-azaheptatriacontane-1,37-dioate) (M03479, Watanabe Chemical Instituted, Hiroshima, Japan), in DMF (0.5 equivalents) was added. The reaction mixture was mixed for 2 h, followed by addition of diethylamine to remove Fmoc. After mixing for 15 min, 0.1 M acetic acid was added to stop the reaction. The mixture was filtered through PTFE membrane filters, and purified by RP-HPLC (phenomenex C18, 4.6φ × 150 mm). Pure fractions were collected and lyophilized to yield dimeric peptide as a white powder.

2.4. Evaluation of phage- or synthetic peptide-binding to recombinant proteins by ELISA

Human FGFR3-Fc was purchased from R&D Systems (MN, USA). Mouse FGFR1c-Fc, mouse KLB-Fc, human KLB-Fc were prepared internally by using a proprietary vector and expression in FreeStyle293 (Thermo Fisher Scientific, MA, USA) cells. The wells of a Nunc Maxisorp microplate (460-518) were coated with an anti-human Fc goat polyclonal antibody (Jackson ImmunoResearch, PA, USA) and blocked with 0.5% BSA in PBS. Fc-fused proteins were captured by the antibody, and phage solution or biotinylated peptide solution was added to the wells. After washing with PBST, bound phages or peptides were detected using horseradish peroxidase (HRP)-conjugated anti-T7 antibody (Merck Millipore, Darmstadt, Germany) or HRP-conjugated streptavidin (VECTOR Laboratories Inc., CA, USA), respectively. The amounts of HRP in wells were measured by detection reagents tetramethylbenzidine (Wako, Osaka, Japan) or a chemical luminescent reagent (Wako, Osaka, Japan).

2.5. Evaluation of agonistic activity of the synthetic peptide in cells

Human primary visceral pre-adipocytes (lot. 8F3482) were purchased from Lonza (Basel, Switzerland) and their differentiation to adipocytes was achieved according to the manufacturer's protocols. The differentiated human primary adipocytes were plated on a poly-D-Lys coated plate and incubated with PGM2 medium (Lonza), respectively. After exchanging the medium with PBM2 containing 0.1% BSA, the cells were starved for 24 h. The peptides diluted with PBS containing 0.1% BSA were added to the wells, and the cells were incubated for 10 min at 37 °C. After removing the

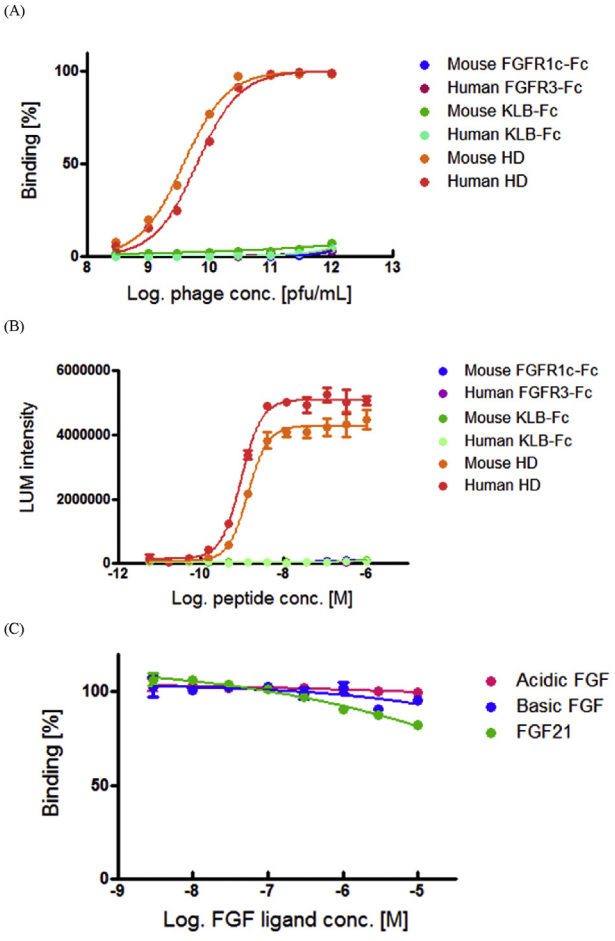

Fig. 1. Binding activity of F91-8A07 phage and its synthetic peptide. (A) Binding activity of F91-8A07 phage to purified recombinant proteins. The binding was detected by anti-T7 phage antibody-HRP and was measured by absorbance at 450 nm. (B) Binding-activity and -selectivity of biotinylated F91-8A07 peptide to purified recombinant proteins. Binding activity was detected by SA-HRP and was measured by chemical luminescence. (C) Ligand competitiveness of F91-8A07 peptide. Vertical line is shown as a relative % value against the binding of biotinylated peptide (1 nM) to human HD in the absence of FGF ligands.

peptide solution, the wells were washed once with PBS and added to cell lysis buffer. The phosphorylated Erk1/2 in cell lysate was measured by Alphascreen (Perkinelmer, MA, USA), according to the manufacturer's protocols.

2.6. Animals

Male C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were maintained under controlled temperature (20–26 °C), humidity (40–70%), and a 12 h light-dark cycle (lights on 7 a.m.) with free access to food (CE-2, CLEA Japan, Inc.) and water. All animal experiments were conducted in accordance with the protocols reviewed by the Institutional Animal Care and Use Committee of Takeda Pharmaceutical Co., Ltd.

2.7. Evaluation of agonist activity of synthetic peptide in vivo

N-(PEG3-LPLP-8A07)$_2$ and FGF21 were dissolved in 10% DMSO/saline. The mice were administered vehicle, N-(PEG3-LPLP-8A07)$_2$ (10 nmol/kg, 100 nmol/kg, 1000 nmol/kg) or FGF21 (10 nmol/kg) subcutaneously (n = 5), and the tissues were excised under anesthesia 1 h after injection. Total RNA was isolated from the liver and brown adipose tissue (BAT) using QIAzol reagent (QIAGEN, Hilden, Germany) and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed using the ABI Prism 7900 Sequence Detection System with TaqMan Universal PCR Master Mix (Applied Biosystems). Mixtures of primers and probes (Applied Biosystems) were used for the detection of mRNA encoding early growth response 1 (Egr-1) protein. The primer and probe sets of cyclophilin D were synthesized, and the primers were ggtcctggcatcttgtccat (forward), cagtcttggcagtgcagataaaa (reverse), and ctggaccaaacacaaacggttccca (probe) (Applied Biosystems). The Egr-1 expression levels were normalized to those of cyclophilin D. Data are expressed as mean ± standard deviation (SD). Statistical significance between vehicle and FGF21 was analyzed using the Student t-test or Aspin-Welch test, and dose-dependency of N-(PEG3-LPLP-8A07)$_2$ was analyzed using the one-tailed Williams test.

3. Results

3.1. Isolation of FGFR1c/KLB-selective binding phage

The mechanism of FGF21-receptor activation is not entirely clear, but previous studies suggested that acceleration of FGFR1c/KLB complex formation and subsequent dimerization of FGFR1c are important [18]. We hypothesized that the molecules binding to interface of FGFR1c/KLB complex would have a potential agonist activity. However, as shown in a previous report, the interaction between FGFR1c and KLB is significantly weak in vitro [16], suggesting that such an interface would be unstable for phage panning. Therefore, we constructed a pseudo-complex: FGFR1c-Fc/KLB-Fc heterodimer (HD) by fusion of extracellular domains of FGFR1c and KLB to the Fc region of Immunoglobulin-G. The HD bound to FGF2, as well as KLB-Fc (data not shown). After 5 rounds of panning random peptide displayed T7 phage libraries against HD, a total of 752 clones were screened for binding activities to FGFR1c-Fc, KLB-Fc, and HD. Most clones bound equally to KLB-Fc and HD, indicating that these clones mainly recognized the KLB region. Others, with the exception of one, showed equal binding to FGFR1c-Fc and HD, indicating that these clones mainly recognized the FGFR1c region.

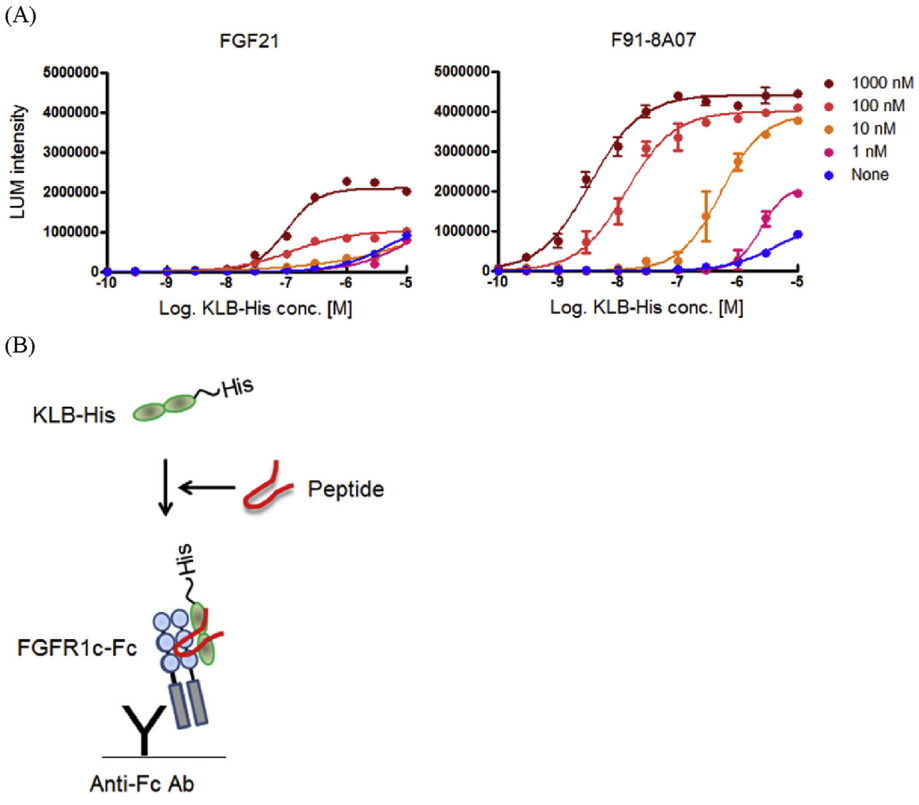

Fig. 2. Binding effect of F91-8A07 peptide on the FGFR1c/KLB complex formation. (A) Effect of F91-8A07 peptide on the interaction between FGFR1c-Fc and KLB-His. The binding of mouse KLB-His to mouse FGFR1c-Fc was evaluated in the absence or presence of human FGF21 or F91-8A07 peptide and detected by anti-His antibody-HRP. (B) Interaction scheme of F91-8A07/FGFR1c/KLB ternary complex.

One phage clone, F91-8A07, bound to both human HD and mouse HD equally, but not to FGFR1c-Fc and KLB-Fc individually (Fig. 1A).

3.2. Binding-activity and -selectivity of synthetic F91-8A07 peptide

We chemically synthesized F91-8A07 sequence (LPGRTCREYPDLWWVRCY) and evaluated its binding activity by ELISA. Biotinylated F91-8A07 peptide demonstrated a phage form and selective binding to HDs, but not to FGFR1c, FGFR3, and KLB individually. The EC_{50} binding values against human HD and mouse HD are 0.9 nM and 1.4 nM, respectively (Fig. 1B). Interestingly, the binding of F91-8A07 peptide to human HD was not inhibited by the presence of human FGF1, FGF2, or FGF21 (Fig. 1C).

Would the F91-8A07 peptide accelerate and stabilize the formation of FGFR1c/KLB complex? To answer this question, we used ELISA to assess the binding between mouse FGFR1c-Fc and mouse KLB-His in the presence or absence of the F91-8A07 peptide. As expected, the binding efficacy and potency of KLB-His to FGFR1c-Fc was dramatically increased in presence of the F91-8A07 peptide (Fig. 2A). This result demonstrates that the F91-8A07 peptide can accelerate the formation of FGFR1c/KLB complex (Fig. 2B), and this ability contributes to its agonistic activity.

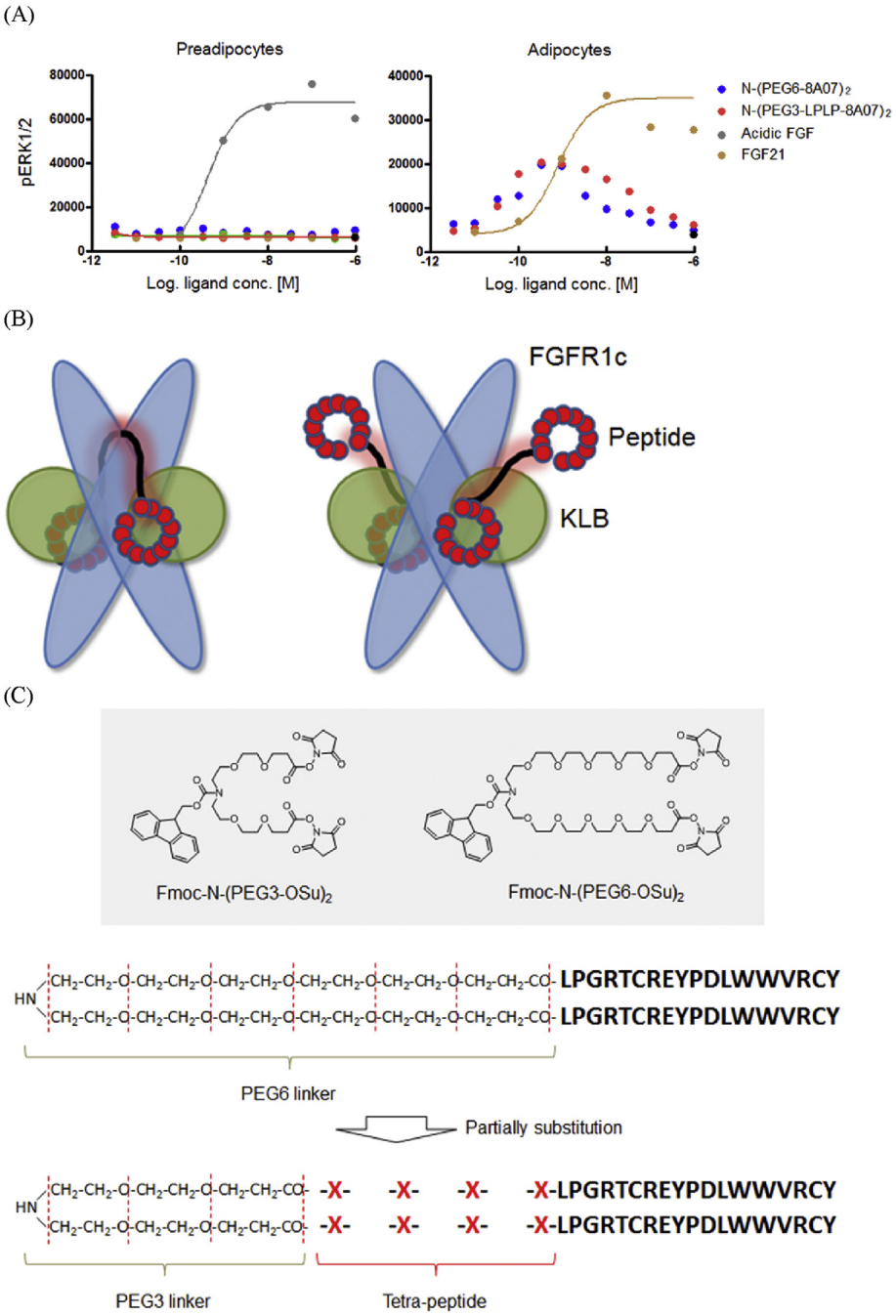

Fig. 3. Agonist activity of dimeric F91-8A07 peptide in primary human adipocytes. (A) Agonist activity of dimeric peptides in cultured primary human visceral pre-adipocytes and adipocytes. Vertical line is shown as counts of total phosphorylated Erk1/2 protein. (B) Interaction scheme of dimeric F91-8A07 peptide with the FGFR1c/KLB complex. (C) Linker design scheme of dimeric F91-8A07 peptide.

3.3. Agonist activity of dimeric F91-8A07 peptide to primary human adipocytes

We evaluated the agonist activity of the monomeric F91-8A07 peptide by measuring phosphorylation of Erk1/2 that is a downstream signal of FGFR1c in primary human visceral adipocytes. Unfortunately, F91-8A07 monomer peptide did not show an agonistic activity (data not shown). Since receptor homodimerization is essential for FGFRs activation, we conjugated N-terminus of the peptide to three chemical linkers of varying lengths (γGlu, PEG3, and PEG6), and constructed dimeric F91-8A07 peptides that would induce tetramerization of the FGFR1c/KLB complex. As expected, the dimeric peptides showed an agonistic activity according to the linker length (data not shown). Of them, PEG6-linked dimeric F91-8A07 peptide showed maximum agonist activity only in adipocytes, but not in pre-adipocytes that do not express KLB (Fig. 3A). In addition, its agonist potency is higher than that of FGF21. This result indicates that peptide dimerization by linkage successfully led to cooperative binding of two F91-8A07 peptides, and induced receptor tetramerization by forming 2:2:2 complex of F91-8A07/FGFR1c/KLB (Fig. 3B).

However, the agonist activity showed a bell-shaped distribution and the intensity was 50%, as compared with that of FGF21 (Fig. 3A). One possible explanation could be binding competitiveness of each dimeric peptide (Fig. 3B), and we predicted that substitution of the long flexible PEG6 linker with amino acids would increase the affinity to FGFR1c/KLB and improve the bell-shaped distribution and partial agonist activity. Accordingly, PEG6 linker was substituted with a PEG3/tetra-peptide hybrid linker (Fig. 3C), and its agonist activity was evaluated. We observed that the resulting N-(PEG3-LPLP-8A07)$_2$ showed relative improvement of bell-shaped agnostic activity (Fig. 3A).

3.4. Agonist activity of dimeric F91-8A07 peptide in vivo

To investigate whether the dimeric peptide behaves as FGFR1c/KLB agonist in vivo, we injected (PEG3-LPLP-8A07)$_2$ to C57BL/6J mice and evaluated the Egr-1 mRNA expression, which is a downstream marker of FGFR1c.We found that N-(PEG3-LPLP-8A07)$_2$ significantly increased the Egr-1 mRNA expression at doses of 10 nmol/kg, 100 nmol/kg, and 1000 nmol/kg in brown fat adipose tissue (BAT), suggesting that our dimeric peptide can activate FGFR1c/KLB in vitro and in vivo (Fig. 4).

4. Discussion

Protein function mimicking peptide, such as an artificial agonist, is useful for not only drug itself, but also as a molecular biology tool. In this report, we successfully generated an artificial peptide agonist to the FGFR1c/KLB complex. The artificial agonists to FGFR1c have been also reported from other companies. For example, Genentech Inc. reported an FGFR1c/KLB bispecific antibody agonist, and Amgen Inc. reported an FGFR1c/KLB bispecific protein, as FGF21-mimicking molecules [19,20]. The point that distinguishes our peptide from the existing ones is the receptor-binding selectivity. F91-8A07 peptide binds to FGFR1c/KLB complex, but not to FGFR1c and KLB individually, suggesting that our peptide recognizes the interface of FGFR1c and KLB. This binding specificity would avoid side-effects when the F91-8A07 peptide is used as a lead molecule for a drug. The binding to FGFR1c that is expressed homeostatically in whole body may induce undesirable side-effects. Also, while the binding to KLB is needed for tissue-selective action, it may lead to a loss of circulating efficacy in vivo, as the molecules will be attached to the KLB expressed in liver. Therefore, the F91-8A07 peptide would access the FGFR1c/KLB complex more effectively, as compared to other FGF21 mimetics.

Where is the binding site of F91-8A07? Interestingly, our peptide did not compete for the binding sites with canonical FGFs and FGF21. As reported, the binding affinity between recombinant KLB and FGFR is in the order of micro molar [16], suggesting that the FGFR1c/KLB complex interface is unstable in its natural state. Therefore, FGF21 could not use the interface and required KLB-binding for its tissue-selective activity in the process of evolution. The discovery of F91-8A07 peptide became possible because we used a pseudo-complex of FGFR1c/KLB with a stable interface. Such an approach will be effective to generate other protein complex-selective peptides.

Dimeric F91-8A07 peptide exhibited agonist activity in primary human adipocytes. Moreover, its potency is higher than that of FGF21. On the other hand, the agonist, as well as the bispecific antibody from Genentech, showed partial activity with a bell-shaped distribution [19]. As we used a flexible PEG linker, competitive binding may occur. Though we could not significantly improve the bell-shaped and partial agonist activity in this report, we consider that further optimization of linker structure and length will be effective. Interestingly, our dimeric peptide significantly induced Egr-1 mRNA expression only in the adipose tissue, but not in the liver, whereas KLB is expressed in the liver and the adipose tissue. Genentech's bispecific antibody also showed agonist effect only in adipose tissues and pancreas, which express high levels of both FGFR1c and KLB, but not in the liver where FGFR1c expression is much lower than that in adipose tissues [19,21]. In contrast, FGF21 induces phosphorylation of Erk even in liver through not only FGFR1c/KLB, but also FGFR3/KLB [22]. These reports support that F91-8A07 peptide accessed the FGFR1c/KLB complex directly

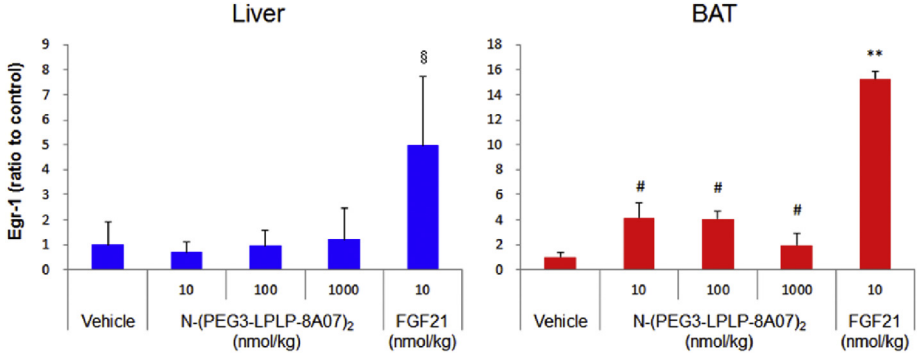

Fig. 4. Agonist activity of dimeric F91-8A07 peptide in vivo. Agonist activity of dimeric peptides in C57BL/6J mice. The expression of Egr-1 mRNA, which is a downstream marker of FGF21, was measured. Data are means + SD (n = 5, $^{\#}P < 0.025$ by Williams test, $^{**}P < 0.01$ by Student's t-test, and $^{\S}P < 0.05$ by Aspin-Welch test).

and activated it in vivo.

Most importantly, we can generate one of the protein functions using a mimicking peptide that can activate FGFR1c/KLB complex by combination of phage display technology and chemical modification. F91-8A07 peptide will contribute to study, drug design, and drug development related to FGFR1c, KLB, and FGF21.

Author contributions

KS conducted most of the experiments, analyzed the results, and wrote most of the paper. YK conducted in vivo experiments. YM conceived and constructed in vitro cell-based assay. TU conducted chemical synthesis of dimeric peptides. TI conducted the recombinant protein preparations. TA, ST, TO, and HI supervised and supported this work.

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